ENZYME-AMPLIFIED REDOX MICROARRAY DETECTION PROCESS

Technical Field of the Invention

The present invention provides a process and an array for assaying for binding of target molecules to capture molecules on microarray devices, wherein the mircoarray devices contain electrodes. Specifically, the present invention provides a binding (including nucleotide hybridization) process to detect binding on a microarray wherein the microarray contains electronically addressable electrode devices. The inventive detection process further provides for an enzymatically catalyzed oxidation/reduction reaction to take place within a "virtual flask" region of a microarray wherein the reaction is detected by current changes detected on the addressable electrode.

Background of the Invention

In the world of microarrays or biochips, biological molecules (*e.g.*, oligonucleotides, polypeptides, oligopeptides and the like) are placed onto surfaces at defined locations for potential binding with target samples of nucleotides or receptors or other molecules. Microarrays are miniaturized arrays of biomolecules available or being developed on a variety of platforms. Much of the initial focus for these microarrays have been in genomics with an emphasis of single nucleotide polymorphisms (SNPs) and genomic DNA detection/validation, functional genomics and proteomics (Wilgenbus and Lichter, J. *Mol. Med.* 77:761, 1999; Ashfari et al., *Cancer Res.* 59:4759, 1999; Kurian et al., *J. Pathol.* 187:267, 1999; Hacia, *Nature Genetics* 21 suppl.:42, 1999; Hacia et al., *Mol. Psychiatry* 3:483, 1998; and Johnson, *Curr. Biol.* 26:R171, 1998).

There are, in general, three categories of microarrays (also called "biochips" and "DNA Arrays" and "Gene Chips" but this descriptive name has been attempted to be a trademark) having oligonucleotide content. Most often, the oligonucleotide microarrays have a solid surface, usually silicon-based and most often a glass microscopic slide. Oligonucleotide microarrays are often made by different techniques, including (1) "spotting" by depositing single nucleotides for *in situ* synthesis or completed oligonucleotides by physical means (ink jet printing and the like), (2) photolithographic techniques for *in situ* oligonucleotide synthesis (see, for example, Fodor U.S. Patent '934 and the additional patents that claim priority from this priority document, (3) electrochemical *in situ* synthesis based upon pH based removal of blocking chemical functional groups (see, for example, Montgomery U.S. Patent 6,093,302 the disclosure of which is incorporated by reference herein and Southern U.S. Patent 5,667,667), and (4) electric field attraction/repulsion of fully-formed oligonucleotides (see, for example, Hollis et al., U.S. Patent 5,653,939 and its duplicate Heller U.S. Patent 5,929,208). Only the first three basic techniques

can form oligonucleotides *in situ* that are, building each oligonucleotide, nucleotide-by-nucleotide, on the microarray surface without placing or attracting fully formed oligonucleotides.

With regard to placing fully-formed oligonucleotides at specific locations, various micro-spotting techniques using computer-controlled plotters or even ink-jet printers have been developed to spot oligonucleotides at defined locations. One techniques loads glass fibers having multiple capillaries drilled through then with different oligonucleotides loaded into each capillary tube. Microarray chips, often simply glass microscope slides, are then stamped out much like a rubber stamp on each sheet of paper of glass slide. It is also possible to use "spotting" techniques to build oligonucleotides *in situ*. Essentially, this involves "spotting" relevant single nucleotides at the exact location or region on a slide (preferably a glass slide) where a particular sequence of oligonucleotide is to be built. Therefore, irrespective of whether or not fully-formed oligonucleotides or single nucleotides are added for *in situ* synthesis, spotting techniques involve the precise placement of materials at specific sites or regions using automated techniques.

Another technique involves a photolithography process involving photomasks to build oligonucleotides *in situ*, base-by-base, by providing a series of precise photomasks coordinated with single nucleotide bases having light-cleavable blocking groups. This technique is described in Fodor et al. U.S. Patent 5,445,934 and it's various progeny patents. Essentially, this technique provides for "solid-phase chemistry, photolabile protecting groups, and photolithography . . . to achieve light-directed spatially-addressable parallel chemical synthesis." Binary masks are used in the preferred embodiment

The electrochemistry platform (Montgomery U.S. Patent 6,093,302, the disclosure of which is incorporated by reference herein) provides a microarray based upon a semiconductor chip platform having a plurality of microelectrodes. This chip design uses Complimentary Metal Oxide Semiconductor (CMOS) technology to create high-density arrays of microelectrodes with parallel addressing for selecting and controlling individual microelectrodes within the array. The electrodes turned on with current flow generate electrochemical reagents (particularly acidic protons) to alter the pH in a small defined "virtual flask" region or volume adjacent to the electrode. The microarray is coated with a porous matrix for a reaction layer material. Thickness and porosity of the material is carefully controlled and biomolecules are synthesized within volumes of the porous matrix whose pH has been altered through controlled diffusion of protons generated electrochemically and whose diffusion is limited by diffusion coefficients and the buffering capacities of solutions.

The microarray systems have detection processes generally using some form of photon-based detection. That is, most detection processes use fluorescent probes (alternatively visible dyes or luminescent probes) attached to "target" DNA sequences to detect binding or hybridization to an oligonucleotide capture probe attached on a microarray. Depending upon the

intensity of the signal, such microarrays having probes to show hybridization have to be read through laser confocal microscope-based system for microarrays configured in a monolayer (such as those microarrays made through high density spotting or photolithography techniques) or by a video-type camera (such as a CCD camera) for those microarrays having a three-dimensional matrix for each spot in high density formats. In each instance, there is often stray light or other noise signals that cause false readings to be made. Moreover, it occasionally becomes difficult to distinguish between shades of gray or barely perceptible signals as true positives or false positives. Therefore, there is a need in the art for improvements to the detection/reading process for analyzing microarrays. The present invention was made to address this need and to provide a detection system that can generate a more objective "yes" or "no" answer for each site in high-density microarray detection.

Summary of the Invention

The present invention provides a process for reading microarray devices having addressable electrodes to determine binding between a capture probe and a target molecule, comprising:

(a) providing an array having a plurality of electrodes and a plurality of capture molecules at sites corresponding to the electrodes;

(b) non-specifically attaching an oxidation/reduction enzyme moiety to one or a plurality of target molecules in a sample for analysis to create a prepped target sample;

(c) administering the prepped target sample to the array and allowing for binding of target molecules to capture molecules;

(d) adding a substrate to the array that will create a local voltage signal when catalyzed by the oxidation/reduction enzyme through local generation of electrochemical reagents; and

(e) measuring for the presence or absence of a voltage or current signal generated locally by electrochemical reagents at each electrode having a capture molecule attached thereto.

Preferably, the array having a plurality of electrodes and capture molecules corresponding to the electrodes is generated by a technique selected from the group consisting of *in situ* synthesis with electrochemical techniques, spotting the capture molecules, ink-jet printing the capture molecules, and *in situ* synthesis through photolighography techniques. Most preferably, the array having a plurality of electrodes and capture molecules corresponding to the electrodes is formed by *in situ* synthesis with electrochemical techniques. Preferably, the oxidation/reduction enzyme is selected from the group consisting of laccase, horseradish peroxidase, β-galactosidase, glucose oxidase, alkaline phosphatase, dehydrogenases, and combinations thereof. Preferably, the oxidation/reduction enzyme is attached to the target molecule(s) through an antibody and anti-idiotype antibody combination or through a biotin and streptavidin (or avidin) binding

combination. Preferably, the array having a plurality of electrodes further comprises a porous reaction layer covering the electrodes, wherein the porous reaction layer has a thickness of from about 0.1 microns to about 10 microns and whereby the porous reaction layer functions to block diffusion of oxidation/reduction activity products such that there is little lateral signal being picked up at an adjacent electrode. Most preferably, the porous reaction layer is made from a polymeric material selected from the group consisting of polyvinyl alcohol, polyvinyl acetate, dextrane, epoxy-based polymers, tricellulose acetate, polyurethane, agarose, controlled porosity glass with a PTFE resin, and combinations thereof. Preferably, the capture molecule is a molecule from the class of molecules selected from the group consisting of oligonucleotides, polypeptides, antibodies, glycosylated polypeptides, polysaccharides, and mixed molecules having monomers from a plurality of the foregoing molecules. Similarly, the target molecule is one likely to bind to at least one of a plurality of capture molecules. Most preferably, a target molecule is from a class of molecules selected from the group consisting of DNA, RNA, single-stranded DNA, ribosomal RNA, mitochondrial DNA, cellular receptors (*i.e.*, glycosylated or non-glycosylated membrane-bound proteins), polypeptides, glycosylated polypeptides, antibodies, cellular antigenic determinants, organic molecules, metal ions, salt anions and cations, and combinations thereof.

The present invention further provides a mircoarray device for detecting binding of a target molecule to a capture probe, comprising:

(a) an array having a plurality of electrodes and a plurality of capture molecules at sites corresponding to the electrodes;

(b) an oxidation/reduction enzymatic moiety bound to one or a plurality of target molecules in a sample for analysis, wherein the oxidation/reduction enzymatic moiety bound to the target molecules is incubated with the capture molecules on the array such that binding between capture molecules and target molecules that bind, will occur;

(c) a substrate molecule that will create a local voltage signal when catalyzed by the oxidation/reduction enzyme through local generation of electrochemical reagents; and

(e) a voltage signal measuring device electrically connected to each electrode on the array.

Preferably, the array having a plurality of electrodes and capture molecules corresponding to the electrodes is generated by a technique selected from the group consisting of *in situ* synthesis with electrochemical techniques, spotting the capture molecules, ink-jet printing the capture molecules, and *in situ* synthesis through photolighography techniques. Most preferably, the array having a plurality of electrodes and capture molecules corresponding to the electrodes is formed by *in situ* synthesis with electrochemical techniques. Preferably, the oxidation/reduction enzyme is selected from the group consisting of laccase, horseradish peroxidase, β-galactosidase, glucose oxidase, alkaline phosphatase, dehydrogenases, and combinations thereof. Preferably, the

oxidation/reduction enzyme is attached to the target molecule(s) through an antibody and anti-idiotype antibody combination or through a biotin and streptavidin (or avidin) binding combination. Preferably, the array having a plurality of electrodes further comprises a porous reaction layer covering the electrodes, wherein the porous reaction layer has a thickness of from about 0.1 microns to about 10 microns and whereby the porous reaction layer functions to block diffusion of oxidation/reduction activity products such that there is little lateral signal being picked up at an adjacent electrode. Most preferably, the porous reaction layer is made from a polymeric material selected from the group consisting of polyvinyl alcohol, polyvinyl acetate, polyvinyl alcohol, tricellulose acetate, polyurethane, agarose, controlled porosity glass with a PTFE resin, dextran, epoxy-based polymers, and combinations thereof. Preferably, the capture molecule is a molecule from the class of molecules selected from the group consisting of oligonucleotides, polypeptides, antibodies, glycosylated polypeptides, polysaccharides, and mixed molecules having monomers from a plurality of the foregoing molecules. Similarly, the target molecule is one likely to bind to at least one of a plurality of capture molecules. Most preferably, a target molecule is from a class of molecules selected from the group consisting of DNA, RNA, single-stranded DNA, ribosomal RNA, mitochondrial DNA, cellular receptors (*i.e.*, glycosylated or non-glycosylated membrane-bound proteins), polypeptides, glycosylated polypeptides, antibodies, cellular antigenic determinants, organic molecules, metal ions, salt anions and cations, and combinations thereof.

Brief Description of the Drawings

Figure 1 shows the chemical reaction scheme when using horseradish peroxidase (HRP) as the oxidation/reduction enzyme. Specifically, the targeted molecule is AGP (α-1 acid glycoprotein) that has been complexed with HRP by adding a biotin-labeled antibody specific for an epitope of AGP. The target molecule is complexed with HRP by adding a avidin-labeled HRP enzyme. The mircoarray site used for detecting AGP as the target molecule has another antibody binding to a different epitope on AGP as the capture molecule. Moreover, the first antibody (labeled "Ab1") is self assembled to an oligonucleotide mircoarray through a tag array capture probe.

Figure 2 shows a similar configuration for detecting AGP as a known site on a mircoarray, except this complex uses the multimeric nature of streptavidin to capture both biotin-labeled second antibody bound to a second epitope on AGP and biotin-labeled different oxidation/reduction enzyme laccase.

Figure 3 shows the chemistry of the oxidation/reduction reaction when β-galactosidase is the oxidation/reduction enzyme and X-Gal (5-bromo-4-chloro-3-indoyl-β-D-ganactopyranoside) is the substrate. The reaction forms indigo blue and generates electrochemical reactants. Preferably,

the reaction is carried out at about pH 7.0 in 0.01 M phosphate buffer (PBS) with 1 mM Fe^{2+}/Fe^{3+}. The X-Gal should be present in saturation amounts.

Figure 4 shows the chemical reaction scheme when using glucose oxidase as the enzyme for oxidation reduction. Electrochemical reagents are generated with PMS (5-methyl-phenazinium methyl sulfate) as the substrate.

Figure 5 shows a reaction scheme with HRP (horse radish peroxidase) as the oxidation/reduction enzyme and catechol as the substrate.

Figure 6 shows a 3D plot of the amperometric measurement of β-galactosidase in an immunoassay detection system. Microarray chips were electrochemically modified with a biotin-containing reagent. In one section of the chip represented by lanes 1-8, no biotin tag was placed. In lanes 9-16, biotinylated β-galactosidase was bound using a biotin-streptavidin-biotin complex. Redox reagents and substrates were added according to the reaction scheme in Figure 3. These data show an amperometric response difference in the two parts of the chip, that is, lanes 1-8 versus lanes 9-16 containing β-galactosidase.

Figure 7 shows a chip profile plot taken from a slice or row of the 3D plot from Figure 6, wherein electrodes 9, 11, 13 and 15 contain β-galactosidase (bound in the region above the electrodes) and register a negative electrochemical signal. Therefore there is a positive result through the inventive electrochemical detection process at spots 9, 11, 13 and 15.

Figure 8 shows binding and detection of a fluorescein-labeled β-galactosidase using an amperometric technique showing redox detection results. The fluorescein acted as a low molecular weight antigen attached to the larger enzyme. The oxidation/reduction enzyme capture was made possible by placement of an affinity-tagged anti-fluorescein antibody on alternating electrodes in row S2. These data show that those known locations associated with electrodes having fluorescein-labeled β-galactosidase showed enhanced signals. Moreover, these data were verified using epi-fluorescent microscopy to confirm the electrochemical results.

Figure 9 shows a slice of a 3D plot created for the capture and detection of glucose oxidase as the oxidation/reduction enzyme. The glucose oxidase specific affinity tags are located in lanes 1-8. The glucose oxidase systems had higher background signals than other oxidation/reduction enzymes.

Figure 10 shows a plot of a voltage versus current display for HRP (horse radish peroxidase) as a redox curve monitored by a single 100 micron diameter electrode on a bare (*i.e.*, no *in situ* synthesis of biomolecules) mircoarray chip. The cyclic voltmogram-like results indicate at a negative potential and the amperometric difference (with and without enzyme) was substantial.

Figure 11 shows the data from Figure 10 in an amperometric experiment performed over a time course in a plot of current versus time for the peroxidase reaction. It should be noted that the current flow levels off after a period of a few minutes.

Figure 12 shows an amperometric 3D plot for rabbit IgG bound to electrodes in S4 (1, 3 and 5) and in S5 (2 and 4) through oligonucleotide tagging. Rabbit IgG was detected by a complex of goat anti-rabbit polyclonal antibody conjugated with HRP oxidation/reduction enzyme. These data are consistent with other data showing that many enzymes function for electrochemical detection.

Figure 13 shows a similar plot of goat IgG bound to electrodes S1 (1, 3 and 5) and S2 (2, 4 and 6) through oligonucleotide tagging. Detection of goat IgG was made by a complex of mouse anti-goat monoclonal antibody conjugated with HRP. Figure 13 reverses the sign of current flow for better visualization.

Figure 14 shows a 3D plot for oligonucleotide hybridization electrochemical detection. Specifically, rabbit and Kras oligonucleotide sequences were *in situ* synthesized on an electrode containing mircoarray device. The chip was set up in an alternating electrode-counter electrode format having a checkerboard pattern of sites having a Kras (or rabbit) oligonucleotide capture probe sequence surrounded diagonally by counter electrodes without oligonucleotides synthesized thereon. Target Kras sample (Operon) was treated to form single-stranded DNA (Operon) and biotinylated with Kras complement (Operon) according to manufacturers instructions. Streptavidin conjugated with HRP (Sigma) was added to the biotinylated Kras sequence complement to form a target complex or complementary Kras affinity-bound to HRP. The target Kras sample complexed with HRP was added to the chip and each electrode was measured for current (amps). These data are shown in Figure 14 in the top panel in a 3D plat and in the bottom panel showing a positive signal in a checkerboard for Kras oligonucleotide capture probes and the bottom panel showing no signal for rabbit sequence captures probes.

Detailed Description of the Invention

There is disclosed a process for reading microarray devices having addressable electrodes to determine binding between a capture probe and a target molecule, comprising:

(a) providing an array having a plurality of electrodes and a plurality of capture molecules at sites corresponding to the electrodes;

(b) non-specifically attaching an oxidation/reduction enzymatic moiety to one or a plurality of target molecules in a sample for analysis to create a prepped target sample;

(c) administering the prepped target sample to the array and allowing for binding of target molecules to capture molecules;

(d) adding a substrate to the array that will create a local voltage signal when catalyzed by the oxidation/reduction enzyme through local generation of electrochemical reagents; and

(e) measuring for the presence or absence of a voltage signal generated locally by electrochemical reagents at each electrode having a capture molecule attached thereto.

Electrode-Based Microarrays

Electrode-based microarrays can be made with various oligomers attached to predefined regions, wherein each predefined region is defined by the presence of an addressable electrode. An addressable electrode is one where it can be electronically accessed to create a current or voltage. Electrode-based microarrays further and often comprise a porous matrix layer that holds the capture molecules and provides a three dimensional virtual flask (cylindrical in the case of a circular electrode). In a preferred embodiment, the porous matrix layer is a membrane, wherein the membrane material is selected from the group consisting of polyvinyl alcohol, polyvinyl acetate, polyvinyl alcohol, tricellulose acetate, polyurethane, agarose, controlled porosity glass with a PTFE resin, and combinations thereof. In each case, the microarray, contains a plurality (on in rare cases only one) of capture molecules. In the most common form of microarray, the capture molecules are oligonucleotides than can bind to complementary sequence regions (or nearly complementary sequence regions depending upon the hybridization conditions) of DNA or mRNA from the target samples. The challenge next becomes how one can detect this binding event or hybridization event. In terms of marketed products, that are generally made by spotting or ink-jet printing oligonucleotides onto planar, non-porous surfaces such as glass slides, there are sample labeling kits commercially available that cause the sample nucleic acid to become labeled with a fluorescent dye. Often it is a fluorescent dye sold under the trademarks of Texas Red®, or Cy® Dyes Cy3 and Cy5. The microarray is "read" through a common fluorometer arrangement with either microscopic magnification or imaging stitching and looking for fluorescence at the known locations where the capture molecule was spotted or synthesized. This common technique of fluorescent detection of microarrays using a standard fluorometer configuration with a microarray is the detection method universally used. However, there are optics issues, difficulty in labeling with fluorescent dyes, occasional high background problems and most importantly, extremely high costs associated with fluorescent microscopic equipment. Therefore, there is a need to detect molecular binding on microarrays using lower cost equipment. The present inventive method uses electrochemical reagents generated locally within a porous reaction layer or membrane to only locally provide current or voltage to a nearby electrode, whose current or voltage signal can be detected at the nearby electrode and not "cross-talk" onto neighboring electrodes.

Immunoassays

Immunoassays are based generally upon antibody binding to another molecule, generally a protein sugar or glycoprotein. The problems of immunoassays are generally detection of this binding event. The sandwich based immunoassays are based upon the fact that one antibody has already been attached to the surface of the chip (the capture antibody; most often a monoclonal). The analyte is then bound to the antibody and a second antibody (usually polyclonal) is added for use as a reporter group. The second reporter antibody will generally contain a fluorophore or have an enzyme covalently attached. Alternatively, the reporter antibody may contain a biotin molecule. To this biotin molecule, a streptavidin-enzyme conjugate can be an attached. Therefore, the inventive process can be constructed with immunoassays, even sandwich-type immunoassays by providing for the oxidation/reduction enzyme to be attached to a complex formed when binding to a capture molecule (*i.e.*, first antibody) occurs. The latter assay formats allows a host of generic assay format to be designed without performing the grueling task of providing (synthesizing) analyte-based individual antibody-enzyme conjugates. Examples of immunoassays in a sandwich configuration are shown in Figures 1 and 2.

Oxidation/Reduction Enzyme Systems

Four different oxidation/reduction enzyme systems are exemplified herein. Each has different potential settings, each function in a unique pH range, they may require redox mediators and lastly, the substrate conditions vary. Reactions schemes are provided for each system.

The HRP (horse radish peroxidase) reaction scheme with catechol as the substrate is shown in Figure 5.

Catechol + H_2O_2 -----→ Quinone + H_2O

Quinone + $2H^+$ + 2e ---→ Catechol.

In a first assay, the product (quinone) was detected amperometrically. The first assay was performed at –0.3 V versus Pt wire in 0.05 M Na-citrate-phosphate buffer pH 5.0 containing 0.2 M Na_2SO_4. Catechol and Hydrogen peroxide in 1 mM concentration were used as an enzymatic substrate. A proper checkerboard pattern from the microarray showed that binding occurred at the proper locations without the presence of noise or cross-talk bleeding over to neighboring electrode locations.

A second assay was performed at –0.3 V vs. Pt wire in 0.05 M Na-citrate-phosphate buffer pH 5.0 containing 0.2 M Na_2SO_4. Catechol in 1 mM concentration was used as an enzymatic substrate. This reaction scheme also produced the expected binding results and followed the reaction scheme as follows:

Catechol + O_2 -----→ Quinone + H_2O

The product (quinone) was detected amperometrically:

Quinone + $2H^+$ + 2e ---→ Catechol.

β-Galactosidase reaction cleaves penultimate β-galactose residues from oligosaccharides or from glycosyl derivatives. The reaction scheme is shown in Figure 3. The substrate used was X-Gal, which is an indolyl derivative of β–galactopyranoside. Biotinylated β–galactosidase was purchased for studies. The reactions were carried out at pH 7.0 in PBS buffer, 0.01 M. A given quantity of X-Gal was dissolved in DMF (very soluble) so that when it was added to the aqueous buffer, a substrate concentration of about 0.1 mM was achieved (saturation). The X-Gal/DMF solution was added while vigorously vortexing the aqueous phase because of the limited solubility of the X-Gal water. If the DMF/X-Gal solution is added without vigorous vortexing, the X-Gal would precipitate from solution. The instability of this solution requires that a fresh solution needs to be prepared daily.

Alternatively, an organic substrate (such as catechol) can be used. The turnover rate for this enzyme is lower than that for other redox enzymes, but the enzyme is very stable at neutral pH.

The X-Gal in itself does not interact well with glass or metal electrodes. Thus, an electron mediator is needed to shuttle electrons to the electrode surface. One such mediator is a ferri/ferro cyanide (50/50) solution. For example, a ferri/ferro cyanide solution can be used at about a 10 mM concentration when using an amperometric reaction and higher or lower concentrations for potentiometric reactions or increasing proportionately with the concentration of the added substrate. The voltage settings used in these experiments were 0 Volts (platinum electrode) and 0.5V for the chip.

The Glucose Oxidase reaction scheme is shown on Figure 4. Preferably, the reaction takes place at about pH 7.5 in a buffer, such as 0.01M PBS buffer. The substrate was glucose and it is extremely soluble in water. The enzyme was regenerated with PMS (5-methyl-phenazinium methyl sulfate), which in turn utilizes the ferro/ferri cyanide shuttle for detection. The potential in this case had the platinum electrode set to 0V and the chip electrode set to 0.5V.

β-Galactosidase Results

Results for beta–galactosidase immunoassay detection system are shown in Figure 6. Microarray chips were electrochemically modified with a biotin-containing reagent. In one section of the chip represented by lanes 1-8, no biotin tag was placed. In lanes 9-16, biotinylated β-galactosidase was bound using a biotin-streptavidin-biotin complex. Redox reagents and substrates were added according to the reaction scheme in Figure 3. These data show an amperometric response difference in the two parts of the chip, that is, lanes 1-8 versus lanes 9-16 containing β-galactosidase. Figure 6 shows a 3D plot of the amperometric reaction with beta-galactosidase. The negative values on this plot indicate that β-galactosidase is bound. The neighboring electrodes are used as counter electrodes in the synthesis process and do not contain

any affinity tags. Similarly, Figure 7 shows this chip profile as a slice (row) of the 3D plot shown in Figure 6. Electrodes 9, 11, 13, and 15 contain beta-galactosidase.

In yet another reaction and immunochemical detection, a capture and detection of fluorescein-containing beta-galactosidase was performed. An anti-fluorescein antibody containing an affinity tag was captured in row two of a biochip made by an *in situ* synthesis electrochemical process (Combimatrix Corporation, Mukilteo, WA). The fluorescein acted as a low molecular weight antigen attached to the larger enzyme. The redox detection results are shown in Figure 8. The electrode sites containing the F-β-galactosidase showed an enhanced value. The binding of F-β-galactosidase to the membrane above the respective electrodes was corroborated and confirmed using the epi-fluorescent microscope. These data exactly track the results obtained with the inventive process of detecting current flow at the electrode. In Figure 8, capture was made possible by the placement of an affinity tagged anti-F antibody on alternate electrodes in row S2.

Glucose-Oxidase Electrochemical Detection

The glucose oxidase enzymatic reaction has some advantages and drawbacks relative to beta-galactosidase enzymatic reaction. The advantages are that all components of this reaction are extremely soluble in aqueous buffer. However, an electron mediator is required, such as PMS. PMS tends to "air oxidize" over time and must be made up freshly or stored under appropriate inert conditions or it will give a higher background signal. In an example of an assay performed with glucose oxidase, biotin was attached to glucose oxidase. The enzyme was then captured on the upper portion of the chip containing streptavidin (Figure 9). As with β-galactosidase above, the chip contained biotin. Electrodes 1-8 contained a first affinity tag (*e.g.*, biotin) and electrodes 9-16 contained a second affinity tag (*e.g.*, streptavidin). The biotin complex allows the capture and detection of the enzyme system. Figure 9 shows a slice of a 3D plot created for the capture and detection of glucose oxidase. The glucose oxidase specific affinity tags are located in lanes 1-8.

Horse Radish Peroxidase Detection

HRP is a redox enzyme that catalyses the reduction of peroxide. The enzyme is small (~ 36 kD) and the enzyme turnover is large. However, peroxide may damage certain polymeric porous matrices or membranes.

$$2I^- + 2H^+ + H_2O_2 \xrightarrow{\text{HRP}} I_2 + 2H_2O$$

$$I_2 + 2e^- \longrightarrow 2I^-$$

The redox curve for the peroxidase reaction was monitored by a single 100 micron diameter electrode on a bare (*i.e.*, no porous membrane or capture molecules synthesized thereon) electrode-containing microarray device and is shown in Figure 10. The cyclic voltamogram like

results indicate at a negative potential, the amperometric difference with and without HRP enzyme was substantial. Taking these data, an amperometric experiment was performed over a period of time (Figure 11). It should be noted that the current flow leveled off after a period of time. Thus, the best time to begin these studies after about 2 minutes (Figure 11).

For the peroxidase system, we have undertaken some initial experiments involving immunoassays. Rabbit IgG or goat IgG were affinity tagged and bound to the upper section and lower section of the membrane, respectively. Detection was made possible using HRP-tagged goat anti-rabbit Ab or HRP-tagged mouse monoclonal anti-goat antibodies. The results of these experiments are shown in Figures 12 and 13. Specifically, Figure 12 shows amperometric detection of rabbit IgG bound to selected electrodes on a microarray biochip containing electrodes and a polyvinyl alcohol porous matrix (Combimatrix Corporation, Mukilteo, WA). HRP-tagged goat anti-Rb was used for the electrochemical detection. Samples are in S4 (1, 3, 5) and S5 (2, 4). The sign of the current flow has been changed for presentation of these data. Further, Figure 13 shows detection of goat IgG bound to selected electrodes on a microarray biochip containing electrodes and a polyvinyl alcohol porous matrix (Combimatrix Corporation, Mukilteo, WA). HRP-tagged mouse monoclonal anti-goat antibody was used for the electrochemical detection. Samples are in S1 (1, 3, 5) and S2 (2 ,4, 6). The sign of the current flow has been changed for presentation of these data. These data are in excellent agreement as to what would be expected based upon earlier work using fluorescent tagged antibodies and standard fluorescence detection.

Oligonucleotide Capture

Figure 14 shows a 3D plot for oligonucleotide hybridization electrochemical detection. Specifically, rabbit and Kras oligonucleotide sequences were *in situ* synthesized on an electrode containing mircoarray device. The Kras sequence used was TACGCCTCCA GCTCC [SEQ IN NO 1]. The rabbit sequence used was AGGCTACGAA GACTT [SEQ ID NO 2]. Therefore, the oligonucleotide capture molecules synthesized by in situ electrochemistry techniques has a sequence of GGAGCTGGTG GCGTA [SEQ ID NO 3] for Kras known locations and a sequence of AAGTCTTCGT CGTAGCCT [SEQ ID NO 4] for rabbit known locations.

The chip was set up in an alternating electrode-counter electrode format having a checkerboard pattern of sites having a Kras (or rabbit) oligonucleotide capture probe sequence surrounded diagonally by counter electrodes without oligonucleotides synthesized thereon. Target Kras sample (Operon) was treated to form single-stranded DNA and biotinylated with Kras complement (Operon) according to manufacturers instructions. Streptavidin conjugated HRP was added to the biotinylated Kras sequence complement to form a target complex or complementary Kras affinity-bound to HRP. The target Kras sample complexed with HRP was added to the chip and each electrode was measured for current (amps). These data are shown in Figure 14 in the top panel in a 3D plat and in the bottom panel showing a positive signal in a checker board for Kras

oligonucleotide capture probes and the bottom panel showing no signal for rabbit sequence capture probes.

Multiple Analyte Detection

The ability of microarray devices to have synthesized many different capture molecules at different known locations allows for multiple analyte detection on a single chip. In each case the sample or samples to be investigated is labeled with an oxidation/reduction enzyme through standard conjugation means. Multiple samples can be pooled so that all of the targets to be investigated can be found from a single pooled sample. In one experiment samples of AGP, ricin and rabbit mRNA samples were pooled and investigated on a single chip using laccase as the oxidation/reduction enzyme. Only those known locations having the appropriate capture molecules detected target even though the group of targets were either protein or nucleic acids. Based upon multiple microarray investigations, the limits of detection were found to be 5 pg/ml for AGP and 300 pg/ml for ricin that translates to 2.5fM in a volume of 0.5 ml. Moreover, the dynamic detection range spanned four logs.

Sequence Listing

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